



## Characterization of *Chromobacterium violaceum* Isolated from Spoiled Vegetables and Antibiogram of Violacein

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**Abstract:** *Chromobacterium violaceum*, a gram-negative, facultative anaerobic, non-spore forming coccobacillus, producing a natural antibiotic called violacein was extracted from *Chromobacterium violaceum* MTCC 2656, collection of Chandigarh. The present study reports different types of microbial population along with *Chromobacterium violaceum* in 36 samples of spoiled vegetables. In the numerous microbial populations, three different strains (HMCCC 45, HMCCC 46 and HMCCC 47) were isolated and subjected to various morphological and biochemical tests. The results reveal that the three strains isolated, HMCCC 46 was similar to the standard except that HMCCC 46 colonies were dark violet in colour. The antibiotic sensitivity test of HMCCC 46 revealed that it was resistant to vancomycin, ampicillin, linezolid, and susceptible to colistin, oxacillin, gentamicin, norfloxacin, chloramphenicol, amikacin.

**Keywords:** *Chromobacterium violaceum*, Purple Colony, Violacein, Antibiotic Susceptibility.

### 1. Introduction

*Chromobacterium violaceum* (Neisseriaceae) is a facultative anaerobic, gram-negative bacillus, first reported from wet rice paste (Boisbaudran, 1882). They are abundant in different latitudes like tropical and subtropical regions and is resident of different sources like spoiled vegetables, soils and freshwater. *Chromobacterium violaceum* associated diseases are unusual because it is an opportunistic bacterium. Hence it has been implicated in system infections particularly tropical latitudes (Richard, 1993). The most distinguished characteristic of *Chromobacterium violaceum* is the production of the purple pigment known as “violacein” (Bromberg and Duran, 2001). Even though it is an opportunistic pathogenic bacterium, its notable product violacein has been introduced as a therapeutic compound for dermatological purposes (Caldas *et al.*, 1978).

In this juncture, the present study has been attempted to characterize the violacein from

*Chromobacterium violaceum* in spoiled vegetables and to assess the antibiotic potential of violacein against the commercially available antibiotic discs.

### 2. Materials and Methods

#### 2.1 Collection of samples

Five samples of spoiled radish and six samples of spoiled brinjal were collected from the different local markets of Coimbatore, India. 10 samples of spoiled radish and 15 samples of brinjal were directly obtained from the producers in different rural areas around Coimbatore. All these samples were collected aseptically in sterile poly-bags kept in the icebox and transported to the laboratory for analyses.

#### 2.2 Screening of violacein producing bacteria

10 grams of each sample were homogenized with 90ml of 0.85% sterile physiological saline (w/v) in a homogeniser for 1 min, followed by the homogenized samples were serially diluted from 10<sup>-1</sup> to 10<sup>-8</sup>. The

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diluents were plated onto nutrient agar plates by spread plate technique and incubated at 40°C for 24 hrs.

Bacterial colonies which produce pigment were isolated, plated and detected in the Luria Bertani agar medium. The screened organisms were cultivated in MRS agar (HiMedia, India) supplemented with 1% CaCO<sub>3</sub>. Purple pigment producing bacteria were screened from the MRS agar plates and the plates were counted by using plate count agar (HiMedia, India) incubated aerobically at 40°C for 48-64 hrs. The purity of the screened colonies was checked by streaking again and subculturing on fresh agar plates of the isolation media, followed by microscopic examinations. Purified strains were preserved in Luria Bertani slant using 15% glycerol (v/v) at 15°C for further studies.

### 2.3 Phenotypic Characterization

Cell morphology and motility of isolates were determined in a phase contrast microscope (Olympus CH3-BH-PC, Japan) following the method of Harrigan (1998). The isolates were subjected to the Gram-staining and tested for catalase production. Preliminary identification and grouping was done on the basis of morphology of the colonies evaluated in Luria Bertani agar medium, based on the ability to grow at different temperatures viz., 30, 40 and 50°C and to grow in buffer liquid medium with different pH conditions like 4.0, 5.0, 6.0 and 7.0 (Hungria *et al.*, 2001). Further, the isolated strains were subjected to phenotypic characterization, determination of the sugar fermentation pattern, other fermentative activity against sugars (mannose, fructose, sucrose, lactose and mannitol), urease test, indole test, methyl red and Voges-Proskauer test, triple sugar iron test, catalase test, oxidase test and citrate utilization tests (Cappuccino *et al.*, 2004).

### 2.4 Production media

The nutritional requirement of *C. violaceum* is necessary to increase the violacein production. Rettori and Duran (1998) has developed the medium containing 0.5% D-glucose, 0.5% peptone, 0.2% yeast extract for the production of violacein pigment. The suspension of the isolated *C. violaceum* was inoculated into 500ml of Rettori and Duran liquid medium and incubated at 40°C for 14 hours at 100 RPM.

### 2.5 Extraction of violacein

After the incubation, the cells were harvested for the extraction and purification of violacein pigment. The purification of violacein was carried out by the collection of the aliquot of 100ml broth after 35 hrs incubation, followed by centrifugation at 10,000g for 10 min. The supernatant was eliminated and 30ml of commercial ethanol (Merck, India) was added to the pellet to extract the pigment from the cells. Further, it was again centrifuged at 8000g for 10 min to separate the cells and the purification was performed by

following the procedure of Duran *et al.*, (1994). Violacein obtained was dissolved in absolute ethanol and 0.004% of dimethyl sulfoxide (I) (Merck, India) and stored at 4°C to protect from the light.

### 2.6 Antibiotic sensitivity test

Phenotypically characterized strains were tested against various commercially available antibiotics to determine the antibiotic sensitivity ranges in comparison with *Chromobacterium violaceum* MTCC 2656 strain collected from the Microbial Type Culture Collection (MTCC), Chandigarh. The antibiotic sensitivity test was accomplished for isolated strains by following the methodology of Kirby-Bauer's NCCLS (National Committee for Clinical Laboratory Standards, 2000) modified disc diffusion technique. The antibiotic sensitivity test for isolated strains was performed on Muller-Hinton agar (HiMedia, India) medium containing 0.8% (w/v) agar by using certain commercially available antibiotic discs such as methicillin (15µg), ampicillin (10µg), amikacin (15µg), chloramphenicol (30µg), colistin (20µg), co-trimoxazole (20µg), furazolidone (30µg), gentamicin (15µg), linezolid (20µg), oxacillin (15µg), norfloxacin (20µg) and vancomycin (20µg). The zone of inhibition was measured by comparison to the standard strain *Chromobacterium violaceum* MTCC 2656 after 24 hours of incubation at 40°C. Based on the sensitivity to the respective antibiotic discs, the isolates were characterized.

## 3. Result and Discussion

### 3.1 Screening for pigment production:

In this study, a total of 36 samples of the spoiled vegetables (brinjal and radish) was analyzed. In these spoiled vegetables, the population of bacteria, as well as the *C. violaceum*, was ranged between 10<sup>-5</sup> to 10<sup>-7</sup> CFU g<sup>-1</sup>, while the brinjal samples collected from the farmers shows that the population was less than the 10<sup>-5</sup> CFU g<sup>-1</sup>. In the serially diluted plates, some strains produced pigment and some produced non-pigmented colonies in the same plate. The pigmented colonies show excellent violet pigment producing capability and concurrently it also shows antagonistic activity against other colonies on the same plate. Similar kinds of result were already reported by (Myers *et al.*, (1992) and Sorenson *et al.*, (1985). Based on these characters, three different isolates were screened on different plates and named as HMCCC 45, HMCCC 46 and HMCCC 47.

### 3.2 Identification of the isolates

The screened isolates HMCCC 45, HMCCC 46 and HMCCC 47 shows typical properties of *C. violaceum* such as low convex, violet, smooth, gelatinous colony morphology on the Nutrient Agar medium. The screened strains show motility in both soft-agar and hanging drop methods and there was an absence of

spore formation. The results obtained from the morphological and cultural characteristics reveal that the three isolates are showing same characteristics and differed only phenotypically (Table 1). Hence, the isolate *HMCCC 46* only was taken for further studies.

### 3.3 Biochemical and Antibiotic-sensitivity profiling of the isolate

Biochemical test profiles of strain *HMCCC 46* are listed in Table 2 and Plate 1. The biochemical tests were conducted in accordance with the method adopted for *Chromobacterium violaceum* UCP 1489 (Bailey and Baron, 1994; Koneman *et al.*, 2001).

Table 1. Morphological and cultural characteristics of *HMCCC 45*, *HMCCC 46* and *HMCCC 47*.

| Test                 | <i>HMCCC 45</i><br><i>HMCCC 46</i><br><i>HMCCC 47</i>  |
|----------------------|--|
| Simple Gram staining | Rod-shaped<br>Pink colored   |
| Nutrient agar        | Convex, smooth and violet colonies for <i>HMCCC 45</i> & <i>47</i> .<br>The strain <i>HMCCC 46</i> had dark violet colonies. |
| Hanging drop         | Motile   |
| Soft – agar          | Motile   |
| Endospore Staining   | Absence of Endospore   |
| Growth at 30°C       | Viable   |
| 40°C                 | Viable   |
| 50°C                 | Less feasible  |

Table 2. Biochemical profile of *HMCCC 46* strain.

| S.No. | Test                      | Reaction                  |
|-------|---------------------------|---------------------------|
| 1.    | Indole production         | -                         |
| 2.    | Methyl Red                | +                         |
| 3.    | Voges-Proskauer           | -                         |
| 4.    | Citrate Utilization       | +                         |
| 5.    | Triple Sugar Iron Agar    | Acid Butt, Alkaline slant |
| 6.    | Hydrogen Sulphide         | -                         |
| 7.    | Catalase                  | +                         |
| 8.    | Oxidase                   | +                         |
| 9.    | Urease                    | +                         |
| 10.   | Starch Hydrolysis         | -                         |
| 11.   | Casein hydrolysis         | +/-                       |
| 12.   | Gelatin Hydrolysis        | -                         |
| 13.   | Carbohydrate Fermentation | Ferment Glucose (+/-)     |

(+) Positive, (-) Negative, (+/-) Variable

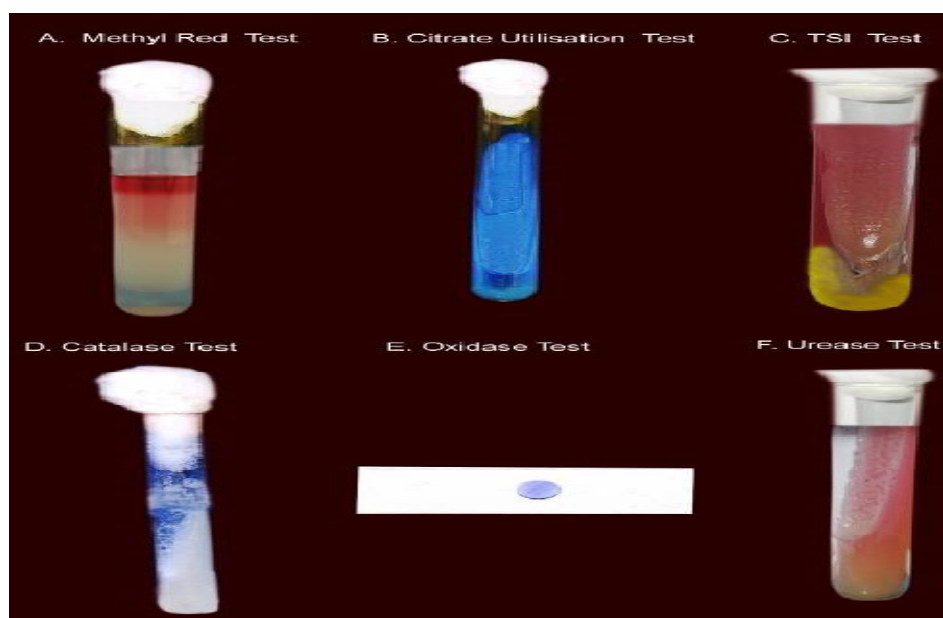


Plate 1. Biochemical profile of *HMCCC 46* strain.

Antibiotic susceptibility test of the isolate was performed by agar disc diffusion methods. The results revealed that the isolate was resistant to vancomycin, ampicillin and linezolid (Table 3). According to Sorenson *et al.*, (1985); Kaufman *et al.*, (1986); Hassan *et al.*, (1993), *C. violaceum* is considered resistant to penicillin, amoxicillin, clavulanate, ampicillin, cephalothin, cefamandole and vancomycin. Resistance prevalence had been always below 60%. In the present investigation, HMCCC 46 strain was susceptible to colistin, oxacillin, gentamicin, norfloxacin, chloramphenicol and amikacin. Intermediary results were obtained for furazolidone, co-trimoxazole and methicillin (Table 3 & Fig. 1).

The results of morphological and biochemical properties of the screened HMCCC 46 strain shows similar characters of *Chromobacterium violaceum* MTCC 2656 (standard strain) obtained from the Microbial Type Culture Collection (MTCC),

Chandigarh. Hence it can be concluded that the strain HMCCC 46 was *Chromobacterium violaceum*.

Table 3. Result of the antibiotic sensitivity of HMCCC 46 strain compared with *C. violaceum* MTCC 2656 (standard).

| Antibiotic      | HMCCC 46 | MTCC 2656 |
|-----------------|----------|-----------|
| Ampicillin      | S        | R         |
| Amikacin        | S        | S         |
| Colistin        | S        | S         |
| Co-trimoxazole  | I        | R         |
| Chloramphenicol | S        | S         |
| Furazolidone    | I        | R         |
| Gentamicin      | S        | S         |
| Linezolid       | R        | R         |
| Methicillin     | I        | I         |
| Oxacillin       | S        | S         |
| Norfloxacin     | S        | S         |
| Vancomycin      | R        | R         |

R – Resistance; S – Sensitive; I – Intermediate

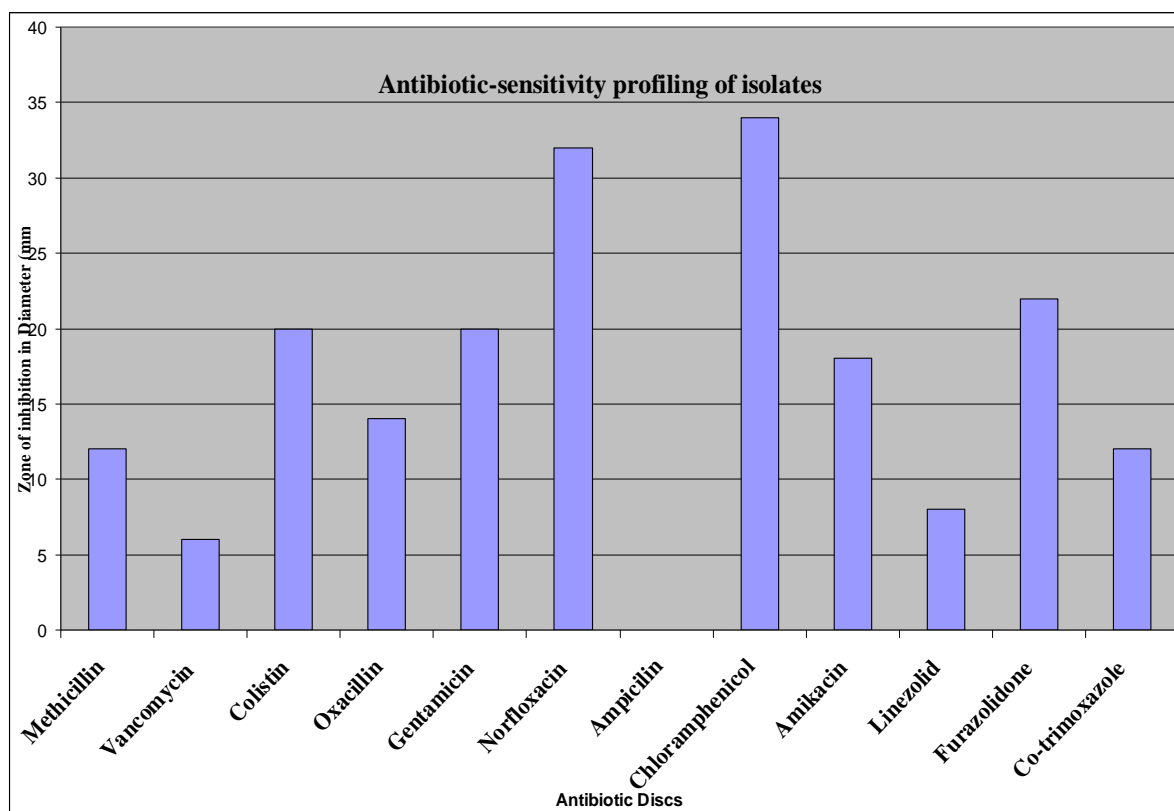


Fig. 1. Antibiotic-sensitivity profiling of isolates.

#### 4. Conclusion

In recent years, emphasis has been placed especially on the chemical nature of the pigments and their probable roles in the metabolism of the bacterial cells. Considerable progress has been made in the solution of these problems. Relatively few workers have published results of research designed to

determine the effects of the physical and chemical environment upon the production of pigments by various species of bacteria. The production of pigment was measured by a gross visual comparison of the amount of pigment of a culture grown upon the test medium with the pigment produced upon a control nutrient agar. The molecular mechanisms coordinating the expression of these characteristics in *C. violaceum*

remain to be determined. Compared to conventional methods, this bacterium leads to an about thirteen-fold yield of pigment.

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